Universidade de Aveiro Departamento de Biologia
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Jesus Domingues

A reporter system to study the role of tRNA modifying enzymes in human proteostasis

Um sistema repórter para estudar o papel das enzimas modificadoras do tRNA na proteostase em humanos

DECLARAÇÃO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ana Raquel Santos Calhôa Mano Soares, Investigadora de pós-doutoramento do Departamento de Ciências Médicas da Universidade de Aveiro e da Doutora Maria de Lourdes Gomes Pereira, Professora associada do Departamento de Biologia da Universidade de Aveiro.

o júri

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Palavras-chave Síntese proteica, Sistema repórter, GFP, HspB1, Proteostase em humanos.

Resumo

A síntese proteica é um processo essencial para que todos os organismos mantenham a homeostasia celular. Os tRNAs são elementos cruciais na síntese proteica, uma vez que codificam a informação genética presente no mRNA.

A linha celular HeLa, utilizada neste estudo, foi primeiramente isolada de uma mulher com cancro do colo do útero e desde então tem sido bastante usada na investigação, sendo muito importante no estudo das bases moleculares de muitas doenças.

De modo a monitorizar a agregação proteica nesta linha celular, um sistema repórter foi desenvolvido utilizando uma fusão entre HspB1 (Hsp27) e a GFP. HspB1 é um chaperone molecular com capacidade de recrutar outros chaperones e restabelecer a conformação ideal das proteínas em situações de stress. A GFP é uma proteína fluorescente que marca certas condições biológicas de interesse.

Para perceber o impacto dos erros da tradução na agregação de proteínas e no surgimento das doenças, o principal objetivo deste estudo foi desenvolver uma linha celular estável (HeLa) expressando um sistema repórter HspB1-GFP, de modo a monitorizar os erros no enovelamento das proteínas em resposta ao *stress* proteotóxico. Ao longo deste estudo o sistema repórter expressando HspB1-GFP foi desenvolvido com sucesso, permitindo assim a sua utilização para identificar situações fisiológicas e patológicas em que a agregação de proteínas ocorre em células de mamífero.

Keywords Protein Synthesis, Reporter system, GFP, HspB1, Human proteostasis.

Abstract

Protein synthesis is essential for all organisms to maintain cell homeostasis. tRNAs are crucial elements in protein synthesis as they decode the genetic information organized in the mRNA codons.

A HeLa cell line, used in this study, was first isolated from a woman with cervical cancer and since then was highly used in biological studies, being extremely important in the study of the molecular basis of several diseases.

In order to monitor protein aggregation in this cell line, a reporter system was developed using an HspB1 (Hsp27) and a GFP fusion. HspB1 is a small heat shock protein that, in stress situations, recruits other proteins in order to restore the conformation of the proteins. GFP is a biosensor that reports several cellular conditions of interest.

To understand the impact of translation errors on protein aggregation and on the disease arising, the main goal of this study was to develop a stable cell line (HeLa) expressing a reporter system HspB1-GFP to monitor the protein misfolding in response to proteotoxic stress. During this study, the reporter system expressing HspB1-GFP was developed successfully, allowing the identification of physiological and pathological situations where protein aggregation occurs in mammalian cells.

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List of Abbreviations

A

A – Adenosine
aaRS – Aminoacyl-tRNA Synthetase
AMP – Adenosine Monophosphate
ATP – Adenosine Triphosphate

C

C – Cytosine

D

dNTP – Deoxynucleotide triphosphate

E

eIF – Eukaryotic Initiation Factor
eRF – Eukaryotic Release Factor
ER – Endoplasmic Reticulum
ERAD – Endoplasmic Reticulum Associated Degradation

G

G – Guanine
GAP – GTPase-activating protein
GDP – Guanosine Diphosphate
GFP – Green Fluorescent Protein
GTP – Guanosine Triphosphate

H

HSE – Heat Shock Element
HSF1 – Heat Shock Factor 1
HSP – Heat Shock Protein
HspB1/Hsp27 – Heat Shock protein 27

M

MAPKAP - mitogen-activated protein kinases associated protein
mRNA – Messenger RNA

P

PABP – Poly(A) Binding Protein
PCR – Polimerase Chain Reaction
PPi – Pyrophosphate
PQC – Protein Quality Control

R

rRNA – Ribosomal RNA

T

TNF-α – Tumor Necrosis Factor α
tRNA – Transfer RNA

U

U - Uridine
UTR – Untranslated Region

Chapter I – Introduction

1.1. Genetic Code

Although Francis Crick believed that the genetic code is universal for all species, as he presented in 1968 (1), some deviations to the code have been identified in some organisms, mainly in organelles. However the molecular basis of the genetic code remains basically the same in all organisms (2).

The genetic code comprises 64 possible three-nucleotide combinations (codons) between the four nucleotides present in the messenger RNA (mRNA), namely, adenosine (A), cytosine (C), guanine (G) and uridine (U). A and G are purines and C and U are pyrimidines (3). However, only 20 amino acids are coded, which indicates the degenerative character of the genetic code. Thus, some amino acids have more than one codon that decodes them and these codons are regularly called synonymous codons (3-5). However, there is only one specific codon that decodes a specific amino acid to initiate the translation mechanism, which is the start codon methionine (AUG) and three specific stop codons (UAA, UAG, UGA) that do not decode any amino acid (2) (Figure 1).

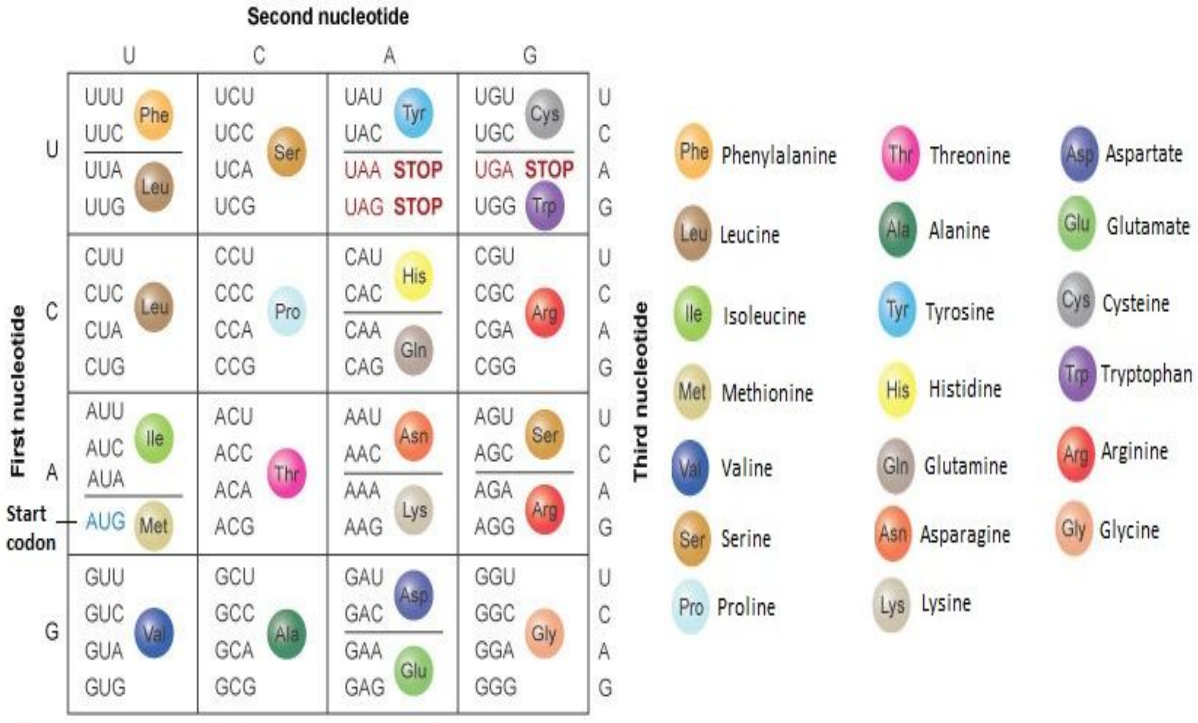


Figure 1. Genetic code. Adapted from Clancy, S. and Brown, W. 2008.

1.2. Eukaryotic Translation

Proteins are involved in all cellular functions, from metabolism and cellular division, to cellular response to environmental stimuli. Thus, maintaining a stable proteome is essential for cell survival (6, 7).

Proteins are the final products of gene expression. This important mechanism that takes place in the cell has two important steps, transcription and translation. In the transcription process, which occurs in the cell nucleus, the information contained in DNA is transferred to a mRNA molecule. This molecule is, later, converted into a protein, in a process called translation or protein synthesis. This process takes place in ribosomes that are ribonucleoproteins located in the cytoplasm of the cells, containing a small and a large subunit, respectively 40S and 60S, according to the sedimentation velocity. These two subunits contain ribosomal RNA (rRNA), transfer RNA (tRNA) and proteins. In the presence of mRNA0 transcripts the small and large subunits join to each other and a tRNA molecule containing a sequence of nucleotide triplets that is complementary to the mRNA, called the anticodon, can pair with the codon. The rRNA facilitates the attachment of a new amino acid to the chain in formation (8).

The ribosome has three sites for tRNA binding, the A-site, P-site and E-site. The aminoacyl-tRNAs are first recruited to the A (amino acid) site where they can base pair with the mRNA codon. The amino acid is transferred from its tRNA to the polypeptide chain in formation on the P (polypeptide) site. The E (exit) site is where binds a free tRNA before being released to the cytoplasm (9).

Translation can be divided in four steps: initiation, elongation, termination and recycling that will be described below (Figure 2).

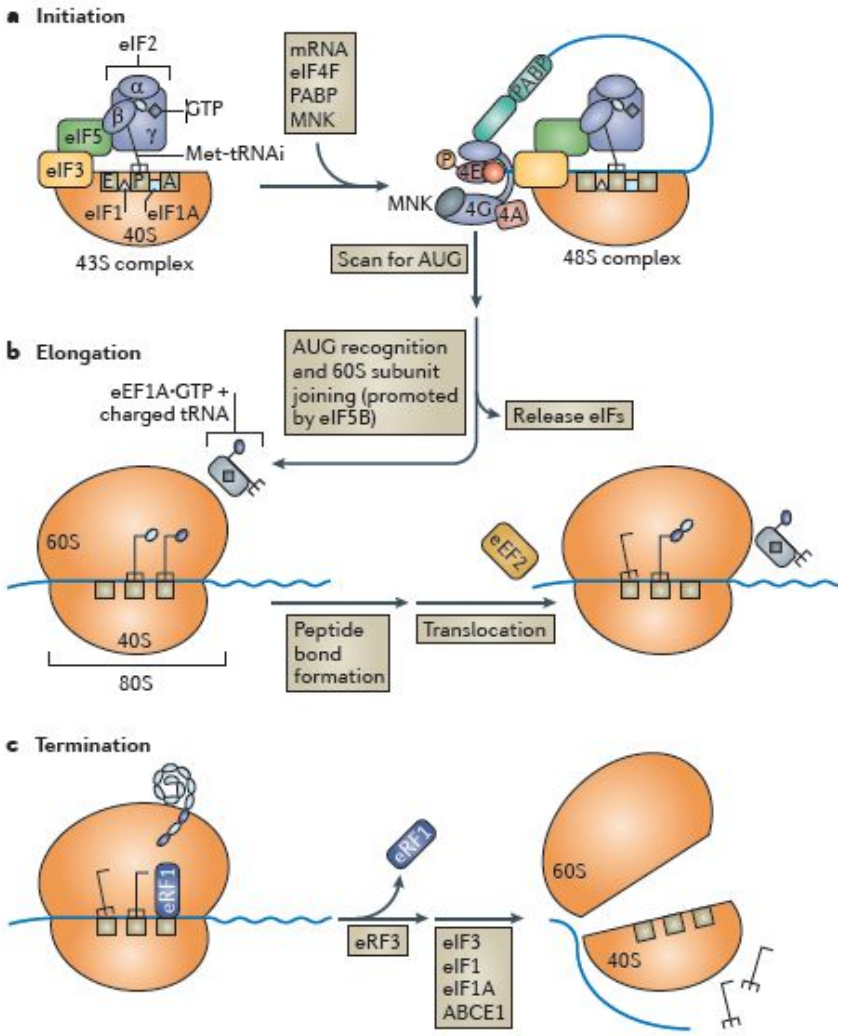


Figure 2. mRNA translation in eukaryotes. **a) Initiation.** Translation begins with a formation of a 43S pre-initiation complex constituted by the 40S ribosome subunit, eIF1, eIF3 complex, eIF5 and the $tRNA^{Met}$. This complex binds to the mRNA, promoting the scanning along the mRNA for the AUG codon. Once founded the start codon, the large ribosomal subunit links to the initiation complex and the next phase can occur. **b) Elongation**. The charged tRNAs are delivered to the A-site of the ribosome and a peptide bond is formed between the methionine and the second amino acid. The ribosome then translocates to the next codon and the polypeptide chain is formed according to the mRNA sequence. **c) Termination.** A stop codon is detected, triggering the release of the mRNA, ribosome dissociation and recycling of the translation components. Adapted from Walsh, D. and Mohr, I. 2011.

1.2.1 Initiation

The initiation of the translation of the mRNA requires the presence of initiation factors (IFs), which bind to the small subunit of the ribosome (10). Translation begins with the formation of a ternary complex that includes eIF2, guanosine triphosphate (GTP) and

methionine-carrying tRNA. Then, the ternary complex binds to the small subunit of the ribosome, which is catalyzed by IF1, IF1A and IF3. The 5' mRNA CAP is recognized and assembled by the eIF4F complex that unfolds structures in the 5' untranslated region (UTR). The eIF4F complex with eIF3 and poly(A) binding protein (PABP) link to the 3' poly(A) tail and bind the mRNA to the ternary complex recently formed, now called 43S complex, allowing the scanning of the mRNA in the 5' to 3'direction (10). When the start codon (AUG) is found, the base pairing between the anticodon of $tRNA^{Met}$ and the initiator codon of the mRNA can occur in the P-site of the small subunit. GTP is hydrolyzed by eIF2 and GTPase-activating protein (GAP) eIF5 and then, eIF2-GDP and other factors, are released from the complex. Once the initiation complex containing the 40S subunit, the mRNA chain and the initiator aminoacyl-tRNA is formed, the large subunit (60S) of the ribosome can link to it, leading to the GTP hydrolysis by eIF5B. Then, the eIF5B-GDP dissociates from the complex, leading to the end of initiation (11-14).

1.2.2. Elongation

In the beginning of elongation the $tRNA^{Met}$ is in the P-site and the A-site is empty. Then, the aminoacyl-tRNA that corresponds to the second codon of the mRNA, either the cognate or noncognate is carried out to the A-site, creating a ternary complex with a GTP and eEF1A (12). Conformational changes on the decoding center of the 40S subunit of the ribosome, hydrolysis of GTP by eEF1A and codon-anticodon interactions ensure the cognate tRNA presence. The eEF1A-GTPase complex is activated, leading to the release of the aminoacyl-tRNA into de A-site by eEF1A-GDP. The resulting eEF1A-GDP must be recycled by eEF1B complex (eEF1Bα and eEF1Bβ) to GTP to be reused in several cycles of polypeptide elongation (8, 11).

The peptidyl transferase center in the large ribosomal subunit, then, catalyzes a peptide bond formation between methionine and the recently added amino acid. The resulting hybrid deacylated tRNA occupies the P-site of the small subunit and its acceptor end is in the E-site of the large subunit of the ribosome. Still, the peptidyl-tRNA has its anticodon end in the A-site of the small ribosomal subunit and its acceptor in the P-site of the large subunit. The next step, catalyzed by eEF2 and GTP, is translocation, where the deacylated tRNA moves to the E-site of the large subunit of the ribosome and the peptidyl-tRNA moves to the P-site. At the same time the mRNA moves three nucleotides towards

the 3' end to put a new codon into the A-site to be decoded. This process is repeated until the detection of a stop codon and the elongation process ends, giving place to the next phase of the translation mechanism (8, 11, 12).

1.2.3. Termination

The termination process occurs when the A-site of the ribosome encounters a stop codon. There are three termination codons: UAA, UAG and UGA that none of the tRNAs recognize (15). Thus, some proteins, namely eukaryotic release factors (eRF) class 1 and 2, decode these stop codons presented in the A-site of the ribosome. eRF2 are GTPases that stimulate the activity of eRF1, which promotes the release of the mRNA from the ribosome and its dissociation with the hydrolysis of the ester bound between the polypeptide chain and the P-site where the tRNA is. So, the components of the elongation complex dissociate, forming separated subunits. The peptidyl transferase center may play a role in this hydrolysis reaction too (8, 11).

1.2.4. Recycling

The last stage of translation is the recycling of the translation components to be further used in another translation cycle. There is lack of information about this mechanism and how this acts in eukaryotic cells. However, the mRNA, the deacylated tRNA and the ribosomal subunits have to be separated and recycled to initiate another translation process (11, 15).

There is a model that suggests that termination and recycling partially dissociate ribosome subunits, in a process called reinitiation of translation. This mechanism results in the shuttle of the 40S subunit across the poly(A) tail to the 5' UTR of the mRNA (15). Some findings suggest that eRF3 and PABP interact to each other to link termination apparatus to the poly (A) tail, being this interaction important for termination and recycling process. However, these findings are still unproven (11).

eIF3 may also have an important role in the recycling process in eukaryotes. Some studies refer that this factor binds to the small subunit of the ribosome and promotes conformational changes, preventing the binding of the large ribosomal subunit, and consequently, promoting the dissociation between these two subunits (11).

1.3. Transfer RNA

tRNAs are adaptor molecules that participate in the translation process. They transport amino acids to the ribosome to decode a mRNA sequence, producing functional proteins (16-19). These RNA molecules, discovered by Paul Zamecnik and collaborators, are the most abundant, representing up to 10% of all RNAs (16, 19). They play an essential role in protein biosynthesis and are able to evaluate the availability in amino acids; however tRNAs participate in several other activities, such as biosynthesis of some metabolites, degradation of proteins, apoptosis and production of tRNA-derived fragments, a type of small interfering RNAs. Their uncharged form has a role in signal transduction pathways in response to nutrient deficiency (16, 20, 21).

tRNAs are composed by 73 to 93 nucleotides with a 3'CCA end in the mature form that is highly conserved among species. tRNAs fold into a cloverleaf in their secondary structure and acquire a L-shaped three dimensional conformation in their tertiary structure (18, 19). The secondary structure is composed by an acceptor arm, a D arm, an anticodon arm and a TΨC arm, which has a variable arm between it and the anticodon arm (Figure 3) (17, 22).

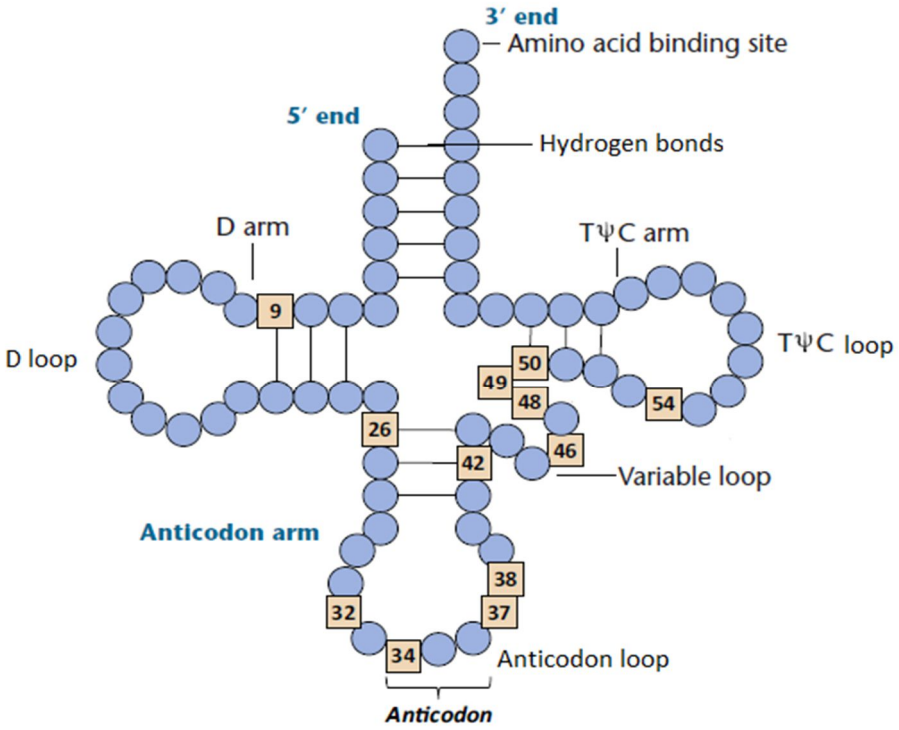


Figure 3. tRNA cloverleaf secondary structure. Adapted from Torres, AG, *et al.* (2014).

The L-shaped tertiary structure is generally conserved and found in almost all tRNA species, providing information about movements in the ribosome throughout translation. The tertiary structure is formed by the stacking between the acceptor and the TΨC arms and between the D arm and the anticodon arm. The D and TΨC loops join together to form the L-shape (16, 17, 19).

After maturation, each tRNA is charged with a cognate amino acid linked to the adenosine in the 3'CCA tail and the mRNA codon base-pairs with the anticodon, reading three nucleotides at a time, at the anticodon loop (17).

tRNA genes are transcribed by RNA polymerase III and to be fully active they have to go through some processes, which includes the removal of extra sequence at the 5' terminus by endonuclease RNase P and the cleavage of the 3' end sequence by endonuclease RNase Z and exonucleases. Then, the post-transcriptional addition of CCA sequence on the 3' end by Transfer RNA nucleotidyltransferases using ATP as substrate. And finally, splicing of introns and post-transcriptional modification of some nucleotides (16, 20).

The mRNA codon is presented to the three bases of the anticodon at positions 34, 35, 36. Watson and Crick in 1966 established that interactions between the first and second positions of the mRNA codon and tRNA anticodon are made according to base-pairing rules that G pairs with C and A pairs with U (23). However, at the third position of the mRNA, the nucleotide can perfectly pair with anticodon or pair with other nucleotides present in the position 34 of the tRNA anticodon, allowing the tRNA to recognize different codons coding for the same amino acid. For example, inosine, which is formed by deamination of A can pair with A, C or U at position 34 of the anticodon, allowing to this wobble hypothesis development. Modifications at position 37 of tRNA stabilize interactions between codon and anticodon, preventing translational frameshift (23, 24).

1.3.1. tRNA Aminoacylation

tRNA aminoacylation reaction is catalyzed by a group of at least 20 enzymes, the aminoacyl-tRNA synthetases (aaRSs). These ubiquitous enzymes control the fidelity of protein synthesis through the attachment of amino acid to their cognate tRNA (25). aaRS have a variety of functions, including transcriptional and translational control, apoptosis inhibition, stimulation of immune system and angiogenic signaling (25, 26).

The aminoacylation reaction occurs in two major steps (Figure 4). In the first step, the cognate amino acid binds to the aaRS, in a process dependent of ATP, leading to the formation of an aminoacyl adenylate (aa-AMP) and releasing of a pyrophosphate (PPi) molecule. In the next step, a tRNA molecule binds to the aaRS complex and the amino acid is transferred to the tRNA, leading to the release of the AMP molecule, the tRNA charged with the cognate amino acid and the aaRS that is now able to start a new aminoacylation reaction (26, 27).

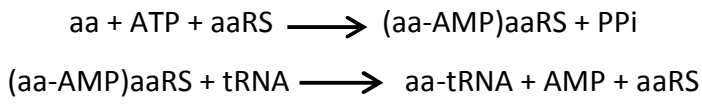


Figure 4. Representation of the aminoacylation reaction. aa, amino acid; aaRS, aminoacyl-tRNA synthetase; PPi, pyrophosphate; AMP, adenosine monophosphate. Tamura, K. 2009.

tRNAs can be charged in three different places of the cell, in the nucleus, cytoplasm and in the mitochondria, where translation occurs. Thus, aaRSs have to be in these cell regions, and to promote this process, nuclear aaRSs are imported to the mitochondria. So, each aaRS can be classified according their localization to charged tRNAs. In humans, 16 aaRS are present in the cytoplasm, where they act, 17 in the mitochondria and 3 are bifunctional, which are able to charge tRNAs in both places (26). aaRS can also be classified into two groups, according to their structural features. Group I have active sites with the Rossman fold (parallel β-sheet nucleotide-binding), whereas group II have antiparallel β-sheets with three motifs in their active sites (19, 26).

To avoid errors in tRNA charging that can affect protein synthesis fidelity, aaRSs have an editing ability, removing incorrect amino acids from tRNAs before they reach the ribosome. This quality control mechanism takes place at the aminoacylation step, in the active site, which differentiate non-cognate from cognate amino acids (28). Editing process can occur before or after the non-cognate amino acid is attached to the tRNA, in a pretransfer editing mechanism or a posttransfer editing, respectively. In pre-transfer editing mechanism, the amino acid incorrectly activated is hydrolyzed before its transference to the 3' end of the tRNA, either by translocation, release or hydrolysis of the active site; whereas post-transfer editing needs the translocation of the 3' end of the mischarged tRNA between the active site and the editing site (29).

Despite these editing mechanisms to control protein synthesis fidelity, mutations in some aaRSs genes can occur and are responsible for the appearing of several diseases, mostly neurodegenerative disorders, characterized by accumulation of misfolded proteins and protein quality control systems saturation (26, 30).

1.3.2. tRNA Modifications

There are more than 600 different sequences of tRNA known so far, with about 120 modified nucleosides, mostly at the anticodon loop, and more than 100 of enzymes required to catalyze these modifications at the post-transcriptional level. Many studies refer that heavily modified tRNAs are more stable than unmodified ones (16, 20, 31). Modifications at position 34 of tRNAs are more associated with decoding, whereas modifications at position 37 of tRNA stabilize interactions between codon and anticodon (24). Modifications at the wobble position are essential for recognition of rare codons. The absence of these modifications, either at the wobble position or at position 37 of tRNA anticodon, promotes translational frameshift and increase levels of missense errors, compromising protein synthesis (32). Some evidences refer that defects on these modifications or in enzymes that catalyze these modifications can trigger the accumulation of misfolded proteins, leading to protein aggregation and to the appearing of some disorders, such as neurodegenerative diseases, cancer and mitochondrial related diseases (33-37) . Therefore these modifications are crucial for translation efficiency as well as tRNA stability, being catalyzed by different groups of tRNA modifying enzymes (Table I) (24).

Table I. Examples of human tRNA modifying enzymes and respective modifications as well as the associated diseases. Adapted from Torres, AG, Batlle E and Ribas de Pouplana, L (2014).

tRNA modifying enzymes	Modifications	Diseases
tRNA methyltransferase 1 (TRM1)	N2,N2-dimethyl guanosines (m^2_2G) at position 26	Cognitive disorders
FtsJ RNA methyltransferase homolog 1 (FTSJ1)	2'O-methylribose at positions 32 and 34	Intellectual disability

NOP2/Sun RNA methyltransferase family member 2 (NSUN2)	5-methylcytosine (m^5C) at positions 34, 48, 49 and 50	Intellectual disorders, mental retardation and cancer
Adenosine deaminase acting on tRNA 3 (ADAT3)	Conversion of adenosine-to-inosine (A-to-I editing) at position 34	Intellectual disability
Elongator Protein 3 homolog (ELP3)	5-methoxycarbonylmethyluridine (mcm^5U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) at position 34	Amyotrophic Lateral Sclerosis (ALS)
Elongator Protein 4 homolog (ELP4)	5-methoxycarbonylmethyluridine (mcm^5U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) at position 34	Epilepsy
tRNA methyltransferase 12 (TRMT12)	Wybutosine at position 37	Breast cancer
DNA methyltransferase 2 (DNMT2)	5-methylcytosine (m^5C) at position 38	Epigenetic cancer therapy
Mitochondrial (mt) $tRNA^{Leu}(UAA)$	5-taurinomethyluridine (tm^5U)	Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)
Mitochondrial (mt) $tRNA^{Lys}(UUU)$	5-taurinomethyl-2-thiouridine (tm^5s^2U)	Myoclonus epilepsy associated with ragged-red fibers (MERRF)

1.4. Protein Folding

Maintaining a stable proteome is essential for cell survival, since proteins are involved in every cellular mechanisms (7). Thus, to function properly, proteins need to fold into its native three dimensional structures, which are characteristic of each protein and involve the action of weak non-covalent bonds between amino acids (38, 39). The

sequences of amino acids have all the information necessary to proper folding, which depends on the amino acid sequence characteristics and on the cellular milieu. In this process several molecular chaperones and proteases participate, to ensure the quality of the final product (40). Although the number of different protein conformations is very large, the protein must acquire the conformation with the lowest energy, despite the native state of some proteins is not necessarily the lowest-energy form. Moreover, correctly folded proteins have stability for long time and can interact with other biological structures in cell environments (41).

In the past years significant efforts were made to understand the process of folding (Figure 5). Some studies refer that, beyond the unfold and the native states, the nascent polypeptide chain, after translation, can also acquire an intermediary compact structure, named molten globule, which is an intermediate configuration. Thus, the protein folding process can be divided in three major steps: fast secondary structures formation, formation of the molten globule and formation of the native protein. The formation of this structures depends on the hydrophobic groups and the heterogeneity of the stabilizing interactions and the native structures are obtained through the hydrogen bonds, van der waals and electrostatic interactions (38, 42).

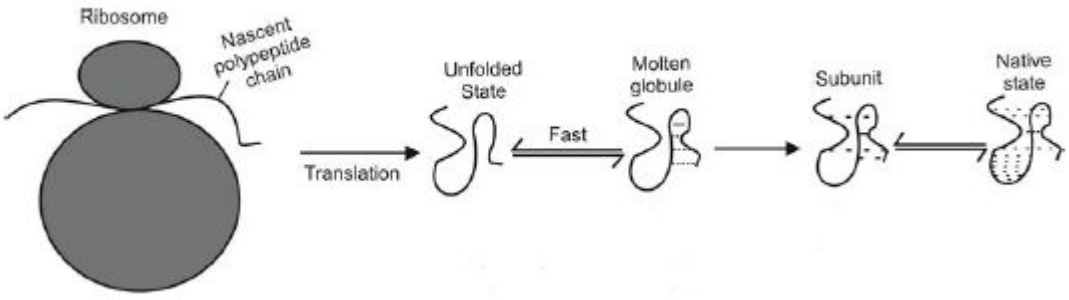


Figure 5. Representation of the folding process. Adapted from Naeen A, Khan TA, *et al*. 2015

The mechanism of folding is initiated, in some cases, after protein translation, when the recently formed chain is still attached to the ribosome. In other cases, protein folding occurs in the cytoplasm of the cell, after ribosome release. And finally, proteins can either fold in specific compartments, such as endoplasmic reticulum (ER) or in the mitochondria.

Although the folding process depends on the environment in which it occurs, the basic mechanisms are still the same (43).

Protein folding forms a large variety of structures that are in tight relation with other important functional groups, promoting the different systems to develop diversity in their chemical mechanisms. Also the folding process is associated with other biological processes, such as, the trafficking of molecules to specific cell compartments, regulation of cell growth, as well as differentiation (43).

The existence of polypeptide chain errors, from gene variations or deficient amino acid modifications, can modify protein folding, resulting in the formation of misfolded proteins and deregulation of protein homeostasis. Accumulation of misfolded proteins and alteration of protein homeostasis are in the basis of the appearance of several diseases, from cancer to neurodegenerative disorders. However, the cell has protein quality controls that are activated to maintain the stability of the proteins, in response to environmental or physiological stress (44, 45).

1.5. Protein Quality Control

A stable proteome is essential for cell stability and survival, because proteins are crucial substances that ensure cell homeostasis. For that, the cell has highly regulated vigilance systems that ensure that proteins are correctly translated and folded, avoiding aggregation and eliminating misfolded and damaged proteins. When cell homeostasis is changed, mostly derived from stress situations, protein quality control systems are activated (6, 7, 44). The protein quality control (PQC) system is constituted by molecular chaperones, intracellular proteases and accessory factors that assist in the folding of the different proteins (44).

Proteins are translocated into the ER, where they acquire their three-dimensional conformation and become functional, before its secretion through the Golgi apparatus. The lumen of the ER is composed by highly concentrations of chaperones and folding enzymes. In this way, the PQC systems need to satisfy a set of characteristics to distinguish folded from misfolded proteins and to avoid the releasing of aberrant proteins into the extracellular environment to exert their function (43, 44). Accumulation of misfolded

proteins is detected by the PQC systems that send these incorrectly folded proteins to refold by molecular chaperones and other folding proteins or, if it is not possible, to degradation by endoplasmic reticulum associated degradation (ERAD) or autophagy (43, 46).

Although the PQC is very important for cell homeostasis maintenance, protein folding and the removal of proteins with aberrant conformation, the existence of errors can interfere with these systems and lead to the appearance of several diseases (44). Aging itself can be another stressing agent that promotes the loss of efficiency of PQC, leading to protein homeostasis imbalance, protein aggregation and disease (7, 44).

1.5.1. Molecular Chaperones

The molecular chaperones are proteins that assist in *de novo* folding or refolding of misfolded proteins. This protein family plays essential roles in protein folding, through the maintenance of the protein complexes in folding competent state, target unfolded or misfolded proteins, translocation across cellular compartments, and direct misfolded proteins to degradation by the proteasome and also have a role in signal transduction (7, 47, 48).

The majority of the members of the chaperones family are designated by heat shock proteins (HSPs), as they are upregulated with the high temperatures and other stress conditions (49). HSPs are classified according to their molecular weight (kDa) in six main families: HSP40, HSP60, HSP70, HSP90, HSP100 and the small HSPs (48-50). In each gene family are chaperones that are constitutively expressed, inducible expressed or both (50).

The heat shock factor 1 (HSF1) is the master transcriptional regulator of the *HSPs* gene expression and is activated by heat, inflammation or other stress conditions that causes the accumulation of misfolded proteins (51). When HSF1 is activated, by phosphorylation, it translocates to the nucleus of the cell and bind to the heat shock elements (HSE), which are located in the promoter regions of target genes, enhancing the expression of the heat shock genes and, therefore, the expression of the HSPs (7, 50, 51).

The Hsp27 also known as heat shock protein beta-1 (HspB1) belongs to the family of the small heat shock proteins due to their low molecular mass (27kDa). This class I small heat shock protein acts through ATP-independent mechanisms and is expressed

constitutively and ubiquitously in most of the cells. The Hsp27 is activated through phosphorylation by several stress conditions, namely heavy metals, oxidative stress, hormones, hypoxia and others (51). This phosphorylation is catalyzed by mitogen-activated protein kinases associated protein kinases (MAPKAP kinases 2, 3), target of the MAP p38 protein kinase (47, 52). Despite their functions as molecular chaperone, Hsp27 is also implicated in modulation of cell growth, differentiation, redox state and tumorigenicity (53, 54). Increased levels of Hsp27 have been implicated in several neurodegenerative disorders and cancers, such as breast and endometrial cancer and leukemia (50), and also mutations are associated with Charcot-Marie-Tooth and distal hereditary motor neuropathy, both characterized by degeneration of peripheral nerves (48). The upregulation of Hsp27 has a role in the protection of the cells against apoptosis by tumor necrosis factor-alpha (TNF-α) (53). Thus, this HSP is involved in many cell functions, being also involved as a potential therapeutic target for several diseases.

1.6. HeLa Cells

HeLa cells are the first human cell line established in culture in 1951 from Henrietta Lacks, a patient with cervical cancer (55). This cell line name comes from the first two letters of the patient's name (56). HeLa cells have important features that enable them to be easily used in laboratory researches. Different from normally somatic cells that undergo senescence, losing the ability to replicate themselves after some generations, HeLa cells divide indefinitely and are resistant to apoptosis. Thus, they are immortalized and easy to culture and maintain (55, 57). Since their discovery, this cell line becomes the most widely used for biomedical researches, being used to evaluate the biochemical pathways involved in normal and disease tissues of human cells (55, 58). Initially, HeLa cells were used to develop the vaccine against polio virus, a human enterovirus that causes poliomyelitis, and since then HeLa cells are used in several areas, from cancer investigations to the mapping of genes, treatment of diseases and mechanisms associated with apoptosis (58). Indeed this cell line is highly used as a model to study biomedical features, being an important tool for biomedical research.

1.7. Reporter Systems

Several biological studies use reporter systems to introduce or select genes of interest in order to monitor their functions in cells. For that, fluorescent proteins in combination with other proteins of interest form reporter systems (59, 60). These fluorescent proteins can serve as probes to monitor characteristics of interest within living cells and, associated with proteins of interest can provide important data about protein's distribution, their dynamics and association with other proteins (60). So, fluorescent proteins can be easily quantified and reflect the expression of a characteristic of interest by enzymatic activity, fluorescence, colorimetric or luminescence intensity (59).

Green Fluorescent Protein (GFP) was discovered and isolated from *Aequorea victoria*, a bioluminescent jellyfish and is the most used fluorescent protein, exhibiting green light when exposed to light in the blue to ultraviolet range (59, 60). GFP folds into a β-barrel of eleven strands, with a helix in the middle. Helical segments cover the ends and help to isolate the internal chromophore (60, 61). GFP can be separated in two segments that fused with two other proteins emit fluorescence. For this protein the folding state is essential for fluorescence emission. Thus, denatured GFP is not fluorescent, restoring the fluorescence upon renaturation and the chromophore alone is also not fluorescent (61). To improve the GFP function, two amino acids were mutated to promote brightness and folding and the resulting protein is called eGFP (enhanced GFP) (60).

1.8. Aim of the Study

Several studies report the implications of translation errors and the accumulation of misfolded proteins in some diseases. In order to clarify this, the main objective of this study was to develop a HeLa stable cell line expressing a reporter system with the fusion of the HspB1 (Hsp27) and GFP, to monitor protein misfolding and proteotoxic stress in human cells. Therefore, to construct the reporter system, this study had the following objectives:

- Construction of a pcDNA3.1 plasmid with the HspB1-GFP promoter and coding sequence;
- Transfection of the pcDNA3.1 with HspB1-GFP in HeLa cells;
- Generation of a HeLa stable cell lines expressing the reporter fusion system HspB1-GFP.

Chapter II –

Material and Methods

2.1. Experimental Model

To monitor protein aggregation in cells, an aggregation reporter expressing HspB1-GFP was developed. HspB1 is a heat shock protein that is recruited in stress situations and binds to misfolded proteins, allowing the action of other heat shock proteins, to refold back the conformation of the protein (62). GFP is the most useful tool in biological studies because has an important function as biosensor to report biological conditions in cellular environment (63). Thus, the cellular localization of this HspB1-GFP fusion reporter fluorescence to foci can identify situations where protein misfolding is increased.

To perform this project a HeLa cell line was used. This cell line was first isolated from a woman who had an aggressive glandular cervical cancer and since then it became an important science instrument implemented in a large amount of researches (56).

A HeLa cell line was transfected with plasmids: pcDNA3.1 containing the reporter fusion system HspB1-GFP, pcDNA3.1 only containing GFP, which is the control in the several assays performed. A negative control with HeLa parental cells was also performed to monitor the transfection of the plasmids. Cells were grown in a specific medium (DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (Pen-Strep)) and, to validate this reporter system, transfected transient cells were incubated after 48 hours with a stressing agent that causes the appearance of stress granules, and might be enriched in misfolded proteins, that could be visualized in the fluorescence microscope. HeLa cells were transfected with the reporter system and stable cell lines were later obtained by Geneticin (G418) selection. G418 resistance gene is included in the pcDNA3.1 plasmid, so only the cells that have incorporated the plasmid are able to grow in the presence of G418. Stable transfected cells were then incubated with a stressing agent at different concentrations to monitor protein misfolding and the appearance of stress granules, in order to validate the stable cell lines expressing the reporter HspB1-GFP.

2.2. Material and Methods

2.2.1. pCS2 Plasmid

To create the reporter fusion system HspB1-GFP, GFP was first amplified from the pCS2 palsmid. This plasmid contains a promoter region of a cytomegalovirus (IE94), a SV40 polyadenylation (PolyA) signal, and an ampicillin resistance gene (Figure 6). A Polimerase Chain Reaction (PCR) reaction mix (Annex 1) was made according to the number of samples and primers tested (Table II). For that, primers for eGFP were used, containing the XhoI and HindIII restriction enzymes, to amplify GFP already with the restriction site overhangs so the GFP could be inserted into the pcDNA3.1 plasmid. In order to amplify GFP with high-fidelity a pfu DNA polymerase was used, because this enzyme has superior thermos-stability than the commonly used, the Taq DNA polymerase. PCR was performed in the MyCycler™ Thermal Cycler (Bio-Rad).

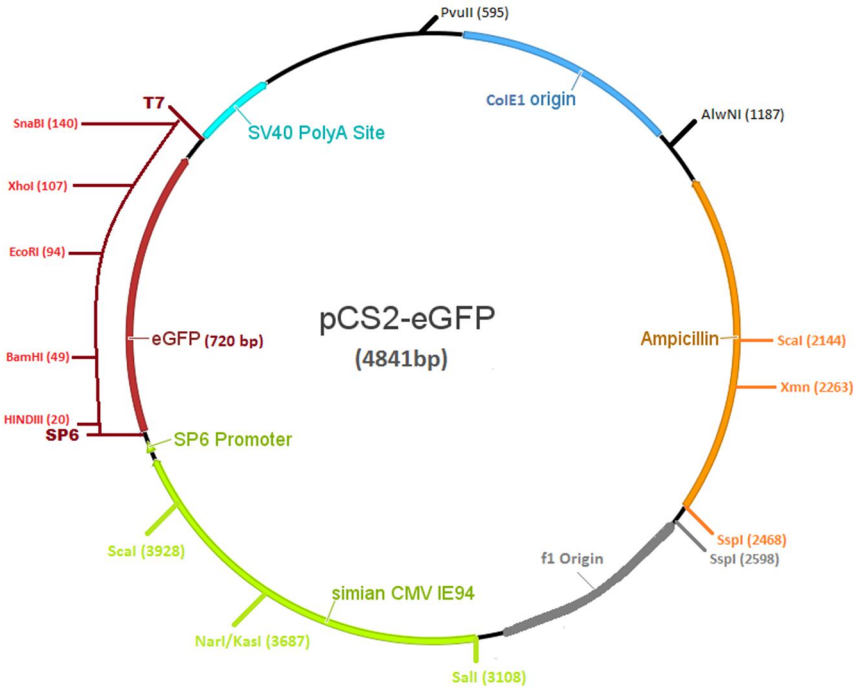


Figure 6. pCS2 plasmid containing GFP coding sequence.

Table II. Primers used in PCR reaction and their sequence.

Primers	Sequence
Forward eGFP XhoI	5' CCGCTCGAGATGGTGAGCAA 3'
Reverse eGFP HindIII	5' CCCAAGCTTTTACTTGTACAGCT 3'

2.2.2. pcDNA3.1 Plasmid

Another plasmid was used, the pcDNA3.1 that already had the human HspB1 promoter and coding region. To clone the GFP amplified from the pCS2 plasmid into the pcDNA3.1 vector (Figure 7), were used HindIII and XhoI restriction enzymes that digest the plasmid in specific regions, allowing the subsequent ligation of GFP. pcDNA3.1 also had the human promoter region, a SV40 promoter and origin, SV40 Poly(A) signal and an ampicillin resistance gene that facilitates the selection of *Escherichia coli* (*E. coli*) competent cells containing the plasmid. Besides ampicillin, G418 is a good marker to select monoclonal populations in stable transfected cells.

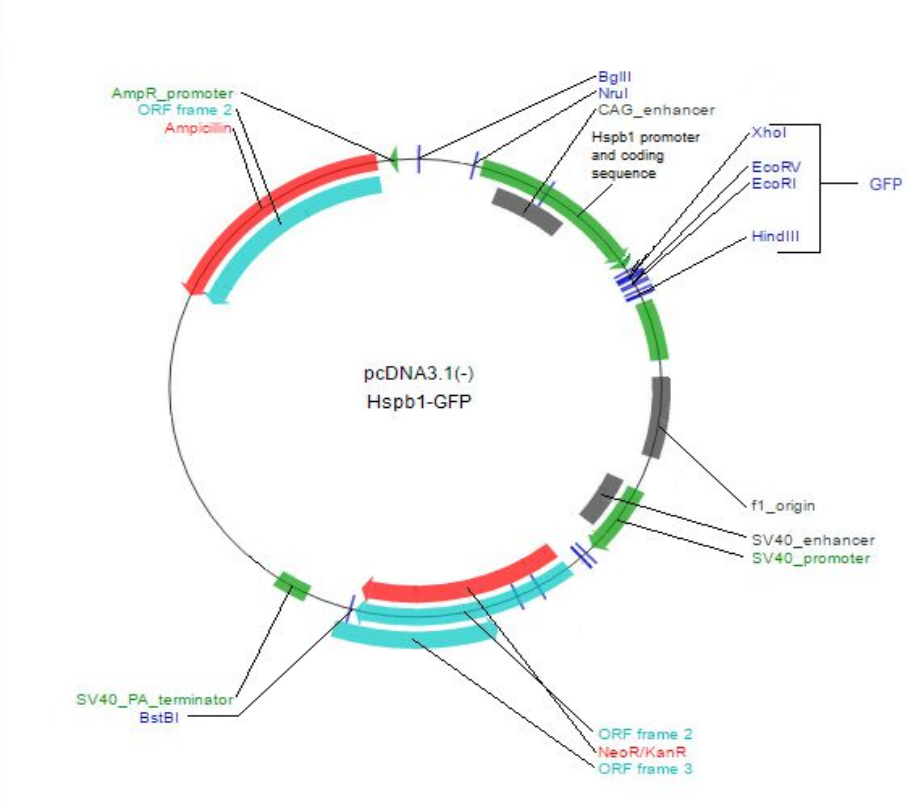


Figure 7. A fluorescence reporter system with the fusion of HspB1 and GFP cloned in pcDNA3.1.

2.2.3. Cloning Assay

A digestion with the XhoI and HindIII restriction enzymes was performed, with different reactions, according to the table III. The PCR product that results from amplification of GFP from pCS2 plasmid was digested with the restriction enzymes to posteriorly be cloned in the pcDNA3.1 plasmid. Also the pcDNA3.1 was digested with the same enzymes, both the pcDNA3.1 plasmid and the pcDNA3.1 HspB1.

Table III. Reaction mix of the digestion with the restriction enzymes.

Reactions	Mix
GFP XhoI and Hind III	R Buffer (10x) - 2µL
	XhoI - 1µL
	HindIII - 1µL
	DNA - 1µg
	Water up to 20µL
pcDNA3.1 XhoI and HindIII pcDNA3.1 HspB1 XhoI and HindIII	R Buffer (10x) - 2µL
	XhoI - 1µL
	HindIII - 1µL
	DNA - 1µg
	Water up to 20µL

These reactions were incubated with XhoI and HindIII restriction enzymes overnight at 37ºC and then treated with FAST AP for 2 hours at 37ºC to avoid the recircularization of the plasmid. The next step was to denature these reactions at 80ºC in the thermoblock to obtain the DNA products. These DNA products were purified using a DNA purification kit (Nzytech), according to the manufacturer's instructions and DNA concentration was quantified using a DeNovix DS-11 spectrophotometer (Frilabo).

Next, the ligation step between the pcDNA3.1 plasmids (pcDNA3.1 and pcDNA3.1 HspB1) and the GFP was performed. For that we diluted the plasmid and the insert to a concentration of 10ng/µL and 4 different reactions were prepared, one for positive control (with the circular plasmid), one for negative control (without the GFP insert) and two to

test ligation efficiency with different ratios (1:3, 1:5), for both pcDNA3.1 and pcDNA3.1 HspB1 (Annex 2). These ligation reactions were left overnight at 16ºC.

2.2.4. Transformation (*Escherichia coli* (*E. coli*) DH5α)

E.coli competent cells were incubated with ligation reactions to incorporate the DNA of the pcDNA3.1 plasmid. For that 10µL of the reactions obtained on the previous step was added to 200µL of *E.coli* competent cells and incubated for 30 minutes on ice, 90 seconds at 42ºC and then left two minutes on ice. 800µL of Super Optimal broth with Catabolite repression (SOC) medium was added and then the reactions were incubated at 37ºC for 1 hour in constant shaking (180 rpm). After that, the reactions were centrifuged for 3 minutes at 4000 rpm and plated in Lysogeny Broth (LB) selective medium supplemented with ampicillin and incubated overnight at 37ºC. Several vials were made with some colonies of transformed *E. coli* cells cultivated in LB medium supplemented with ampicillin, and incubated overnight in constant shaking at 37ºC. These vials were then frozen at -80ºC.

2.2.5. DNA Extraction and Quantification

Nzytech MiniPrep kit was used to extract DNA from the plasmid in transformed *E. coli* cells. Then, DNA concentration was quantified using a DeNovix DS-11 spectrophotometer (Frilabo).

2.2.6. DNA Sequencing

To confirm if the GFP sequence was inserted into the pcDNA3.1 plasmid, the samples were sequenced by STAB VIDA. According to the YOU TUBE IT sequencing service, 3 microtubules with 10µL of the DNA sample at 100ng/µL as well as 3µL of 10µM of the primer, each were prepared. The reactions were:

1 – Plasmid (pcDNA3.1 HspB1-GFP) and an HspB1 Forward primer that binds to the beginning of the HspB1 sequence (HspB1 Forward: 5'AGTTGTATGCCCCAACCCAA3');

2 – Plasmid (pcDNA3.1 HspB1-GFP) and an HspB1 Forward primer that binds to the middle of the HspB1 sequence (HspB1 Forward middle: 5' CGGAAATACACGTGAGTCCT 3');

3- Plasmid (pcDNA3.1 HspB1-GFP) and a GFP Forward primer that binds to the beginning of the GFP sequence (eGFP Forward: 5' ATGGTGAGCAAGGGCGAGGA 3').

The retrieved data was analyzed with BioEdit. A pairwise alignment between two sequences, allowing the ends to slide was performed.

2.2.7. Cell Culture

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% of FBS and 1% of Pen-Strep. Cells were kept in an incubator at 37ºC with 5% CO_2 and 95% of humidity.

For all of the assays cells were detached from the plates with 0,05% trypsin and incubated 5 minutes at 37ºC. To neutralize trypsin effect, DMEM was added and then the cell suspension was centrifuged at room temperature and ressuspended in medium. Cell density was determined using a Neubauer chamber to plate only the desired amount of cells ($2x10^5$ cells/mL) for the assays performed.

2.2.8. Transfection

Transfection was performed to deliver DNA from the different pcDNA3.1 plasmids into HeLa cells. Cells were plated in 6 multi well plates with a density of $2x10^5$ cells/mL and after 24 hours transfection was performed using Lipofectamine® 2000. One hour before transfection, the medium was changed for a medium without antibiotics, as antibiotics can affect the transfection efficiency. HeLa cells were transfected with both pcDNA3.1 GFP and pcDNA3.1 HspB1-GFP, as well as the negative control that only had lipofectamine, without the plasmid. 1µg of the plasmid for the DNA complexes and 2µL of Lipofectamine® 2000 (ratio 1:2) were used. After 7 hours of incubation the medium was changed to a medium with antibiotics and cells were kept in culture for at least 48 hours before any other experiment.

2.2.9. Stable cell lines

Cells were transfected with the pcDNA3.1 HspB1-GFP plasmid and 72 hours later G418 antibiotic was added to select only the cells containing the plasmid. Then, successive dilutions of stable clones were made in a 96 well dishes and only the wells with 1 colony were selected. These 1 colony wells grown in the presence of G418 until confluence was reached and then replated in a 24 well culture dishes, and later in 6 well culture dishes. Once confluence was reached, cells were divided into bigger plates and then part of these stable cells was tested to see if they expressed the plasmid.

2.2.10. Stimulation with Sodium Arsenite

48 hours after transfection cells were incubated with sodium arsenite ($NaAsO_2$) at different concentrations (250μM and 500μM). After 30 minutes of incubation cells were obtained by trypsinization and pellets from centrifugation were taken to perform the several assays. This assay was performed for both transient and stable monoclonal cells generated from the transfected cells.

2.2.11. Fixation and Fluorescence Microscopy

Coated coverslips (Corning™) were placed in 6 well plates to promote cell growing and adherence to the coverslip. After 48 hours and the incubation with or without sodium arsenite, the culture medium was removed and cells were washed 3 times with phosphate buffered saline (PBS). Cells were then fixed with a solution containing 4% of paraformaldehyde and 4% of sucrose and incubated 15 minutes at room temperature. Enough volume of 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI – 0,5μg/mL) was added to cover the coverslip and incubated for 15 minutes at room temperature, protected from the light. Coverslips were washed 3 times with PBS and mounted in a glass slide with Fluoroshield mount media. Slides were dried in the dark and then examined in the Zeiss Axio Imager.Z1 fluorescence microscope.

2.2.12. Polimerase Chain Reaction (PCR)

After DNA extraction from pellets obtained in step 2.2.10 by NZY Tissue gDNA Isolation Kit (Nzytech) and quantification of the DNA concentration, a PCR was performed to verify if the right sequence was correctly inserted into HeLa cells. To amplify the HspB1-GFP and GFP sequences a concentration of 100ng of DNA and two pairs of primers were used, as represented in table IV. A reaction mix was prepared (Annex 3), according to the number of the samples tested, and primers were diluted to a concentration of 10µM. PCR was then performed in the MyCycler™ Thermal Cycler (Bio-Rad), according to the cycle represented in Annex 3.

Table IV. Primers used and their sequence

Primers	Sequence
eGFP Forward	5' ATGGTGAGCAAGGGCGAGGA 3’
eGFP Reverse	5' TTACTTGTACAGCTCGTCCA 3'
HspB1 Forward Middle	5' CGGAAATACACGTGAGTCCT 3'

2.2.13. Agarose Gel Electrophoresis

To verify if the PCR products were amplified correctly, according to their molecular weight, an electrophoresis on a 2% agarose gel was performed. PCR products ran with 6X Loading Dye (Thermo Scientific) in an agarose gel with Green Safe Premium (Nzytech) at 80V, in an electrophoretic container with Tris-Acetate-EDTA (TAE) for around 30 minutes. Gels were then analyzed in the ChemiDoc™ XRS+ System (Bio-Rad) with the Image Lab™ software.

2.2.14. Protein Extraction and Quantification

To obtain total protein extracts, pellets were resuspended in 100µL of ELB protein lysis buffer. This buffer broke cell membranes, allowing the study of their content. Protein extracts were then sonicated for 2 cycles during 15 seconds each and centrifuged 20 minutes at 200g and 4ºC. In the end, supernatants were kept for the next phase of total

protein quantification. During all procedures cells should remain in ice, avoiding the activity of proteases.

Quantification of total protein was performed using Pierce™ BCA Protein Assay Kit (Thermo Scientific), following the procedures of the manufacturer. After 30 minutes of incubation at 37ºC to develop colorimetric reaction, absorbance was measured at 575nm in a microplate reader and results were analyzed.

2.2.15. Western Blot

Western blot is an important technique that allows the identification of specific proteins. This technique separates proteins according to their molecular weight through sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, these proteins are transferred to a solid membrane and incubated with specific primary and secondary antibodies to target proteins of interest (64).

A concentration of 20 µg of total protein was loaded in the 10% polyacrylamide gel, according to the quantity measured in the BCA Protein Assay Kit. Samples were prepared with 6X SDS Protein Loading Buffer and then denatured at 95ºC for 5 minutes in constant shaking. Gel was loaded with a molecular marker (Nzy Colour Protein Marker II) in the first well followed by the samples, and then SDS 1X Running Buffer was added to the electrophoretic container to immerse the running gel. SDS-PAGE ran for about 2 hours at 80V in the first 15 minutes, until samples pass through the stacking (upper part of the gel), and then at 100V the rest of the time. Finished the SDS-PAGE, gel proteins were transferred to a 0.2µm nitrocellulose membrane in the Trans-Blot® Turbo™ Transfer System for 7 minutes and blocked 1 hour at room temperature with Tris-Buffered Saline-Tween (TBS-T) with 5% of Bovine Serum Albumine (BSA), to avoid unspecific bonds. After blocking, membranes were incubated with primary antibody for two hours (anti-GFP, Clontech, dilution 1:1000) or overnight (anti-HspB1, StressMarq, dilution 1:1000) at room temperature or 4ºC, respectively. After removal of primary antibody, membranes were washed 3 times with TBS-T, incubating 5 minutes between washes, and then incubated with anti-mouse secondary antibody (IRDye 800 Li-CoR Biosciences, dilution 1:1000) for 1 hour at room temperature, protected from light. Membranes were washed 2 times with TBS-T, 5 minutes each and 1 with TBS for 5 minutes.

Membranes were scanned in an Odyssey Infrared Imaging System (LI-COR, Biosciences Inc.) and results were analyzed in the Odyssey software (LI-COR, Biosciences inc.).

Chapter III –

Results

3.1. Plasmid Construction

3.1.1. Plasmid Amplification

Two different plasmids were constructed, pcDNA3.1 GFP and pcDNA3.1 HspB1-GFP, as described in methods section. After *E.coli* transformation, the DNA from the different clones of the plasmids was extracted and quantified and a PCR to amplify GFP, in order to verify if the cloning was successful, was performed. The results are shown in Figure 8, revealing the presence of the GFP in all of the *E.coli* colonies.

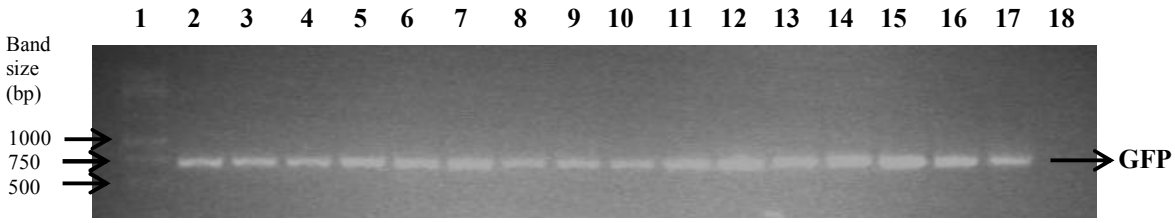


Figure 8. PCR amplification of pcDNA3.1 in transformed *E.coli* competent cells. Lane 1: Ladder; Lane 2 to Lane 9: GFP amplification from clones 1 to 8 of pcDNA3.1 GFP; Lane 10 to Lane 17: GFP amplification from clones 1 to 8 of pcDNA3.1 HspB1-GFP, using Forward and Reverse eGFP primers. Lane 18: negative control.

3.1.2. Plasmid Sequencing

The samples were sequenced by STAB VIDA to confirm if the HspB1-GFP fusion was correctly inserted into the pcDNA3.1 plasmid. As the sequence program used only have the fidelity of 700 bases, 3 reactions were made to sequence the whole HspB1-GFP fusion reporter. According to the sequencing results in Supplemental data, the plasmid pcDNA3.1 contains the HspB1 and GFP sequences, as the three reactions used can pair with the original HspB1-GFP sequence.

3.2. Validation of the reporter system in the HeLa cells

3.2.1. Transfection

A transient transfection was performed to incorporate the pcDNA3.1 GFP and the pcDNA3.1 HspB1-GFP into the HeLa's genome in 6 well plates, as shown in Figure 9. And 48 hours after the cells were stimulated with different concentrations of sodium arsenite to induce stress.

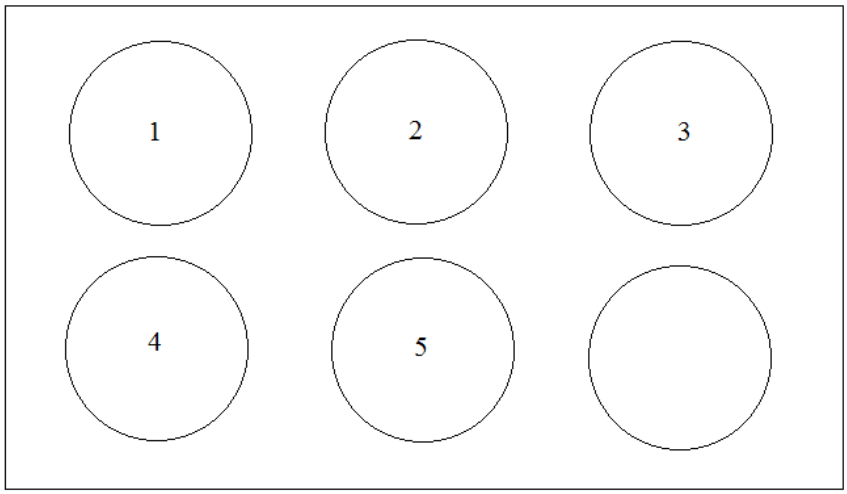


Figure 9. HeLa cells transient transfection with pcDNA3.1 GFP and pcDNA3.1 HspB1-GFP. Well 1: HeLa parental; Well 2: HeLa transfected with pcDNA3.1 GFP; Well 3: HeLa transfected with pcDNA3.1 HspB1-GFP; Well 4: HeLa transfected with pcDNA3.1 HspB1-GFP and incubated with sodium arsenite at 250µM; Well 5: HeLa transfected with pcDNA3.1 HspB1-GFP and incubated with sodium arsenite at 500µM.

3.2.2. PCR

To ensure that HeLa transient cells incorporate properly the plasmid, a PCR was performed. These results confirm the integration of the pcDNA3.1 plasmid, both containing GFP and HspB1-GFP, in HeLa's genome, through the utilization of two different pairs of primers, one for the amplification of GFP (A) and another pair to amplify HspB1-GFP (B), as shown in Figure 10.

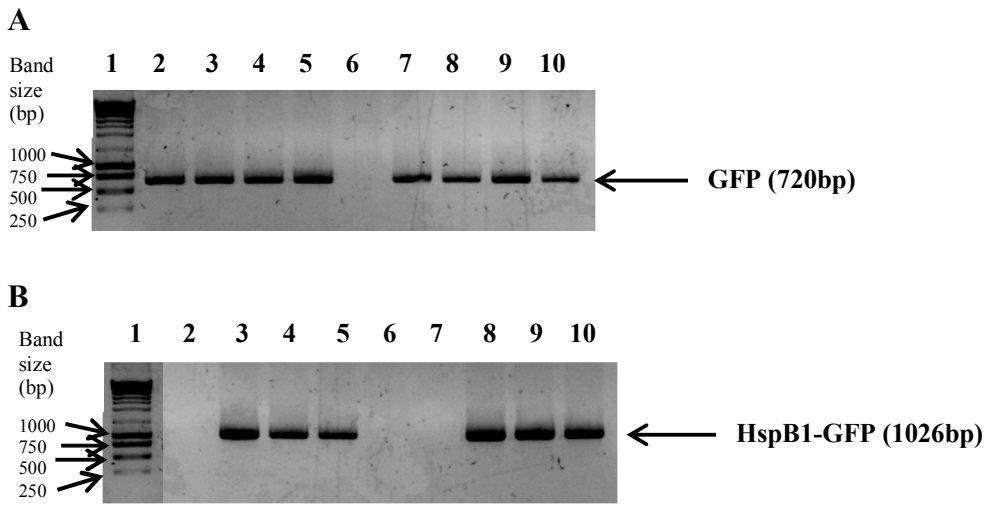


Figure 10. (A) GFP PCR amplification and (B) HspB1-GFP PCR amplification from HeLa cells. Lane 1: Ladder; Lane 2: pcDNA3.1 GFP replica 1; Lane 3: pcDNA3.1 HspB1-GFP replica 1; Lane 4: pcDNA3.1 HspB1-GFP with sodium arsenite at 250µM replica 1; Lane 5: pcDNA3.1 HspB1-GFP with sodium arsenite at 500µM replica 1; Lane 6: Negative control replica 2; Lane 7: pcDNA3.1 GFP replica 2; Lane 8: pcDNA3.1 HspB1-GFP replica 2; Lane 9: pcDNA3.1 HspB1-GFP with sodium arsenite at 250µM replica 2; Lane 10: pcDNA3.1 HspB1-GFP with sodium arsenite at 500µM replica 2.

3.2.3. Fluorescence Microscopy

HeLa cells were transiently transfected with pcDNA3.1 GFP and pcDNA3.1 HspB1-GFP. GFP is a fluorescent protein, so 48 hours after transfection the integration of the plasmid in HeLa cells can be visualized by fluorescent microscopy. In Figure 11 the integration of the pcDNA3.1 HspB1-GFP (A) and the nucleus marked with DAPI (B) are shown. In Figure 12 the integration of pcDNA3.1 HspB1-GFP in HeLa cells (A) and the nucleus marked with DAPI (B) are shown. These cells in Figure 12 were incubated with sodium arsenite at the concentration of 500µM to test the appearance of stress granules in cells, as indicated by the arrows.

A B

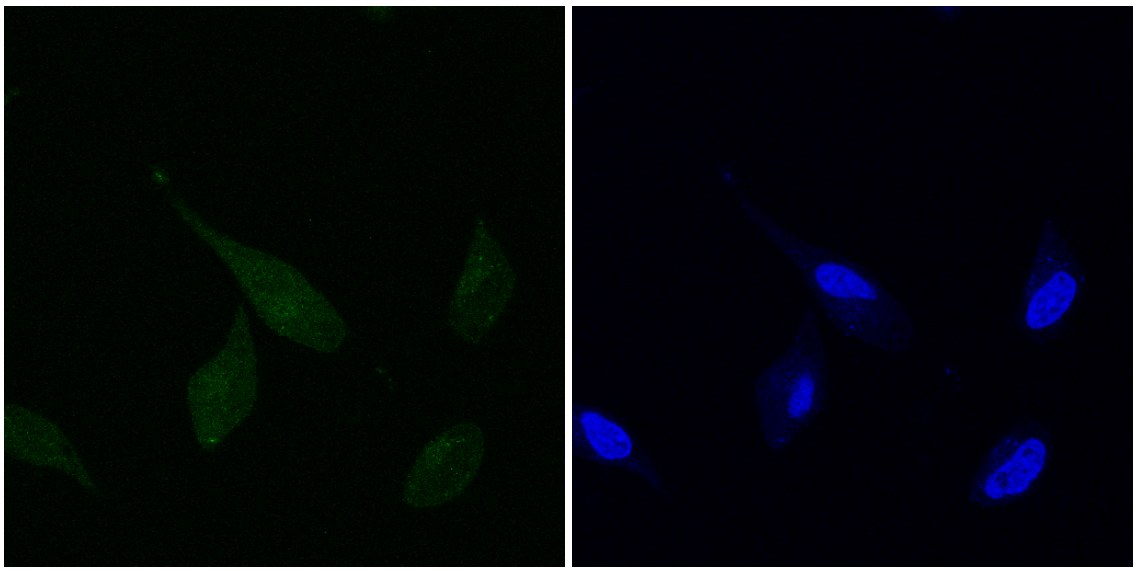


Figure 11. HeLa cells transiently transfected with pcDNA3.1 HspB1-GFP, which is the control, and stained with DAPI, to access the cell nucleus. Cells were visualized at the fluorescence microscope with (A) fluorescent GFP probe and (B) DAPI (63x).

A B

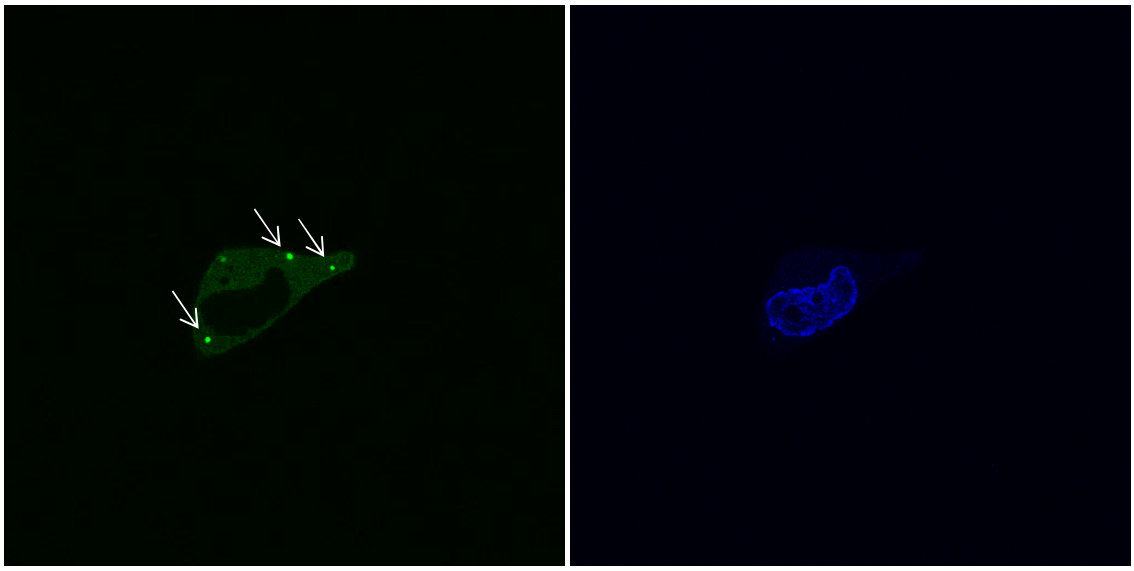
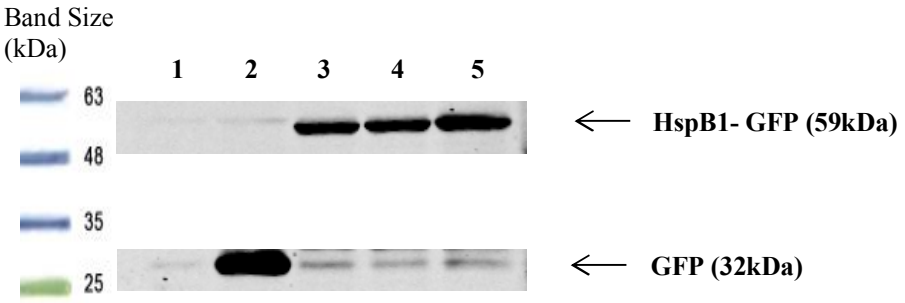


Figure 12. HeLa cells transiently transfected with pcDNA3.1 HspB1-GFP, incubated 30 minutes with sodium arsenite at 500µM and stained with DAPI, to access the cell nucleus. Cells were visualized at the fluorescence microscopy (63x). In (A) were shown the stress granules indicated by the arrows.

3.2.4. Western Blot

To validate the expression of GFP and HspB1 as well as the reporter fusion HspB1-GFP in HeLa transient cells, the western blot technique was also performed. The relative expressions of GFP and HspB1 are presented in Figure 13 (A) and (B), respectively, as well as the respective fusion reporter (HspB1-GFP).

A



B

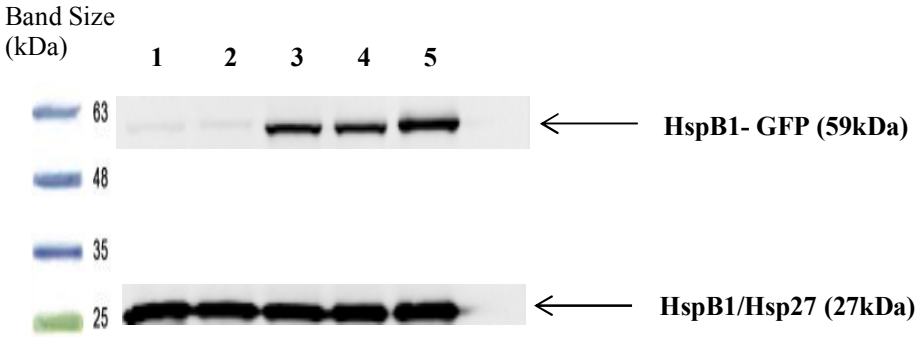


Figure 13. Expression of GFP (A) and HspB1 (B) in HeLa transient cells. Lane 1: HeLa parental cells; Lane 2: HeLa pcDNA3.1 GFP; Lane 3: HeLa HspB1-GFP; Lane 4: HeLa HspB1-GFP with sodium arsenite at 250µM; Lane 5: HeLa HspB1-GFP with sodium arsenite at 500µM. Total protein was extracted from HeLa cell pellets of the different conditions tested, described in the legend, and then 10% polyacrylamide gel was loaded with 20µg of total protein. Proteins were transferred to a nitrocellulose membrane and incubated with the respective antibodies.

3.3. Monoclonal cell lines

3.3.1. Monoclonal Cells Selection

To select only monoclonal strains from HeLa HspB1-GFP stable cells, successive dilutions in 96 well plates were made. Cells were visualized under the optical microscope to monitor their growing and wells with two or more colonies were despised. When one colony wells reach confluence, cells were plated in 24 well plates, and then in 6 well plates. After that, those clones were stimulated with different concentrations of sodium arsenite.

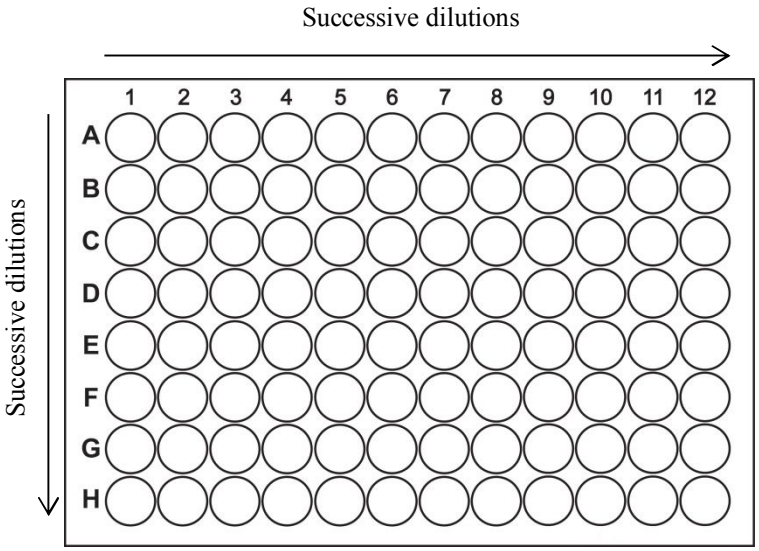


Figure 14. Representation of a 96 well plate used for selection of monoclonal cells of HeLa HspB1-GFP.

3.3.2. PCR

After cell selection with G418, to select only the HeLa cells that express the fusion plasmid, and the selection of monoclonal cells through successive dilutions, the HspB1-GFP fusion was amplified from the four successful clones obtained to verify if those lines were expressing the desired fusion. The incorporation of pcDNA3.1 HspB1-GFP in the stable transfected cells was evaluated by PCR and the results are shown in Figure 15.

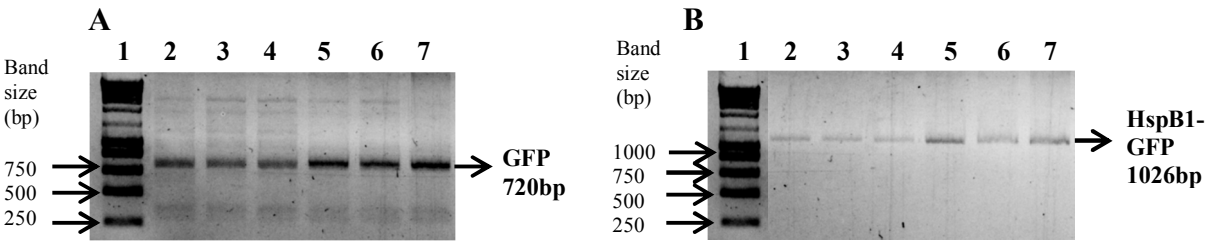


Figure 15. (A) GFP PCR amplification and (B) HspB1-GFP PCR amplification from monoclonal stable HeLa HspB1-GFP cells. Lane 1: Ladder; Lane 2: HeLa HspB1-GFP clone 1; Lane 3: HeLa HspB1-GFP with sodium arsenite at 250µM clone 1; Lane 4: HeLa HspB1-GFP with sodium arsenite at 500µM clone 1; Lane 5: HeLa HspB1-GFP clone 2; Lane 6: HeLa HspB1-GFP clone 3; Lane 7: HeLa HspB1-GFP clone 4.

3.3.3. Fluorescence Microscopy

The integration of the pcDNA3.1 HspB1-GFP plasmid was also evaluated by fluorescence microscopy and the selected clones were studied with or without the influence of the sodium arsenite. Figure 16 shows one of the stable HeLa cell lines expressing the HspB1-GFP control without the action of the sodium arsenite, in A, and with sodium arsenite at concentration of 500µM, in B, which is visible cell shrinkage and the translocation of HspB1-GFP to foci near the nucleus. Figure 17 shows the HspB1-GFP stable cell line control (A) and this stable cell line stimulated with sodium arsenite at 250µM (B), showing cell shrink and the appearance of stress granules.

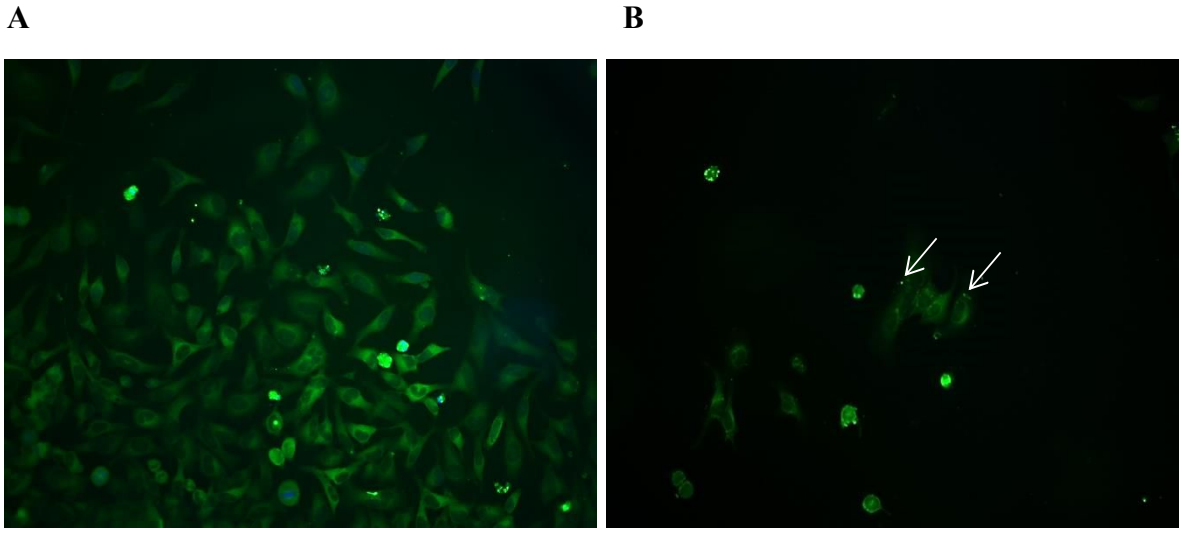


Figure 16. HeLa HspB1-GFP cell line stained with DAPI (20x). In (A) is shown the HeLa HspB1-GFP control and (B) with sodium arsenite at 500µM. White arrows indicate the stress granules.

A B

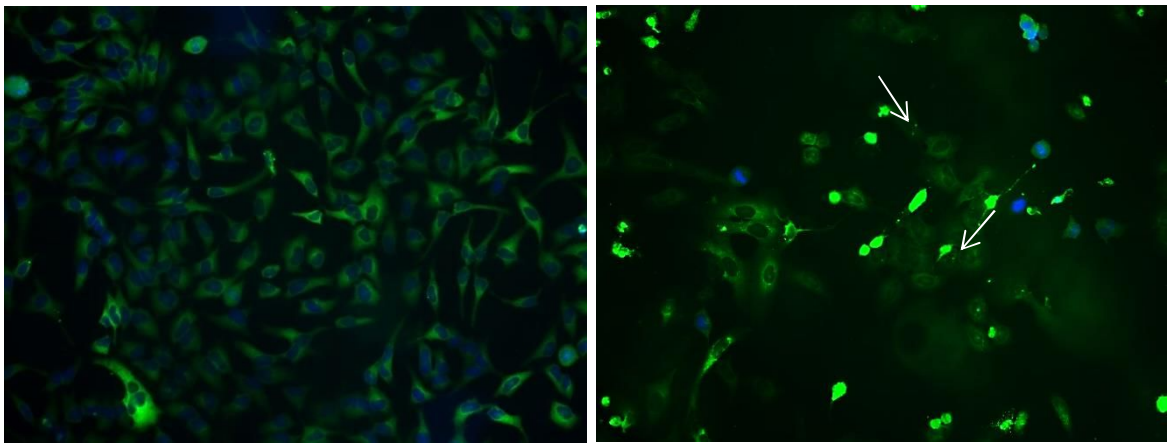
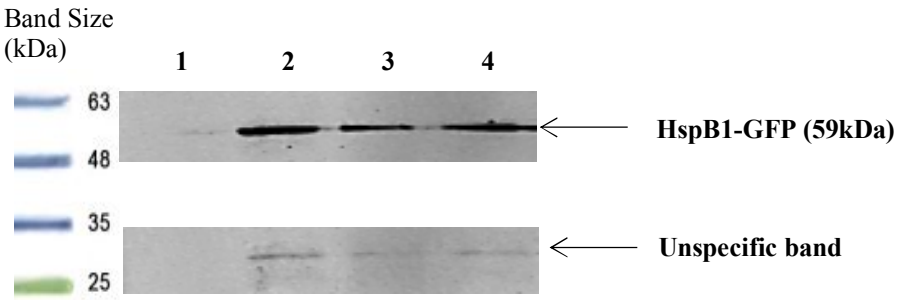


Figure 17. HeLa HspB1-GFP cell line stained with DAPI (20x). In (A) is shown the HeLa HspB1-GFP control and (B) with arsenite at 250μM. White arrows indicate the stress granules.

3.3.4. Western Blot

To verify the expression of GFP and HspB1 as well as the fusion reporter in stable monoclonal HeLa cells, a western blot was performed and their relative expression is revealed in Figure 18. In (A) is presented the expression of GFP and the fusion reporter HspB1-GFP. In (B) is shown the expression of HspB1 as well as the fusion reporter HspB1-GFP.

A



B

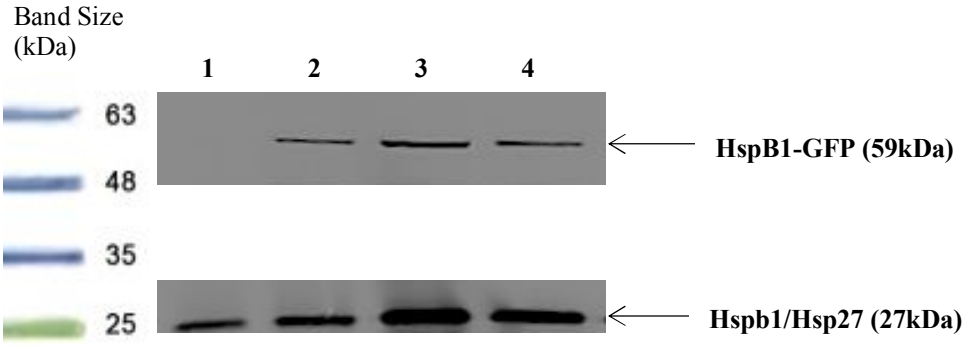


Figure 18. Expression of GFP (A) and HspB1 (B) in stable monoclonal HeLa cells. Lane 1: HeLa parental cells; Lane 2: HeLa HspB1-GFP; Lane 3: HeLa HspB1-GFP with sodium arsenite at 250μM; Lane 4: HeLa HspB1-GFP with sodium arsenite 500μM. Total protein was extracted from HeLa cell pellets with different conditions described in the legend, and then 10% polyacrylamide gel was loaded with 20μg of total protein. Proteins were transferred to a nitrocellulose membrane and incubated with the respective antibodies.

Chapter IV –

Discussion

Conclusion and Future Perspectives

4.1. Discussion

The process of translation is very important for the cell as proteins are involved in every cellular process, and so, the maintenance of a stable proteome is essential for life. However translational errors can occur, leading to mistranslation and protein aggregation, which is reported in some diseases and studies (7, 21, 24, 30). Protein misfolding and aggregation are mostly the cause to the onset of many neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's disease, as well as Amyotrophic Lateral Sclerosis (ALS). Thus, monitoring the protein misfolding and aggregation are a great strategy to understand the molecular basis of these disorders (65).

In this study a reporter fusion system expressing HspB1-GFP to monitor proteotoxic stress and protein aggregation was developed. This reporter can be used in small interfering RNA (siRNA) screenings to identify human tRNA modifying enzymes involved in protein homeostasis, or any other molecule that contribute to protein aggregation.

4.1.1. Plasmid Construction and Sequencing

In this study, a pcDNA3.1 plasmids expressing both GFP and HspB1-GFP were successfully created, as the results of the PCR for plasmid construction show plasmid integration and stability in every *E.coli* colonies, when tested with GFP primers. The HspB1 primers were not tested because HspB1 was already integrated in the pcDNA3.1 for previous studies and the objective was to see if the GFP was properly integrated in the pcDNA3.1 plasmid. The sequencing results corroborate the PCR results, showing proper integration and stability of this reporter system in the plasmid. Although in the PCR were only used GFP primers, sequencing also with the HspB1 primers proved the integration of the whole HspB1-GFP fusion reporter.

4.1.2. Validation of the Reporter System

Fusion proteins are widely used to evaluate the expression of particular features of interest, as the expression of the reporter systems reflects the expression of the characteristics in study. So the reporter systems can be easily quantified and measured (59). In this study a small heat shock protein (HspB1) fused to the GFP, a fluorescence protein, was used to monitor protein aggregation in HeLa cells. First the plasmid expressing HspB1-GFP was developed; and then the HeLa transfection was performed. To verify the fidelity of the transfection, a PCR was performed and to monitor the expression of the reporter fusion system, fluorescence microscopy and the western blot analysis were also performed.

The PCR results from transient transfection of HeLa cells with the pcDNA3.1 GFP and pcDNA3.1 HspB1-GFP show properly integration of both plasmids. To corroborate these results, the HeLa cells were visualized under the fluorescence microscope for protein aggregation evaluation, as cells were stimulated with sodium arsenite at concentrations of 250μM and 500μM. Fluorescence microscopy results showed the appearance of stress granules in the HeLa cells transiently transfected with pcDNA3.1 HspB1-GFP and stimulated with sodium arsenite at 500μM, when compared to these cells without the action of sodium arsenite. The results also revealed the localization of this fusion reporter HspB1-GFP to the nucleus of the cell.

In response to the environmental stimuli, the cells activate mechanisms to prevent damage. One of these mechanisms is the formation of stress granules that are aggregates of the translation preinitiation complexes. The formation of the stress granules leads to inhibition of the translation and the synthesis of HSPs and enzymes for stress response (66, 67). The HSPs, namely HspB1, have an important role on regulation of the stress response and on the maintenance of the cell homeostasis, preventing protein aggregation (68, 69). A study in Rat-1 cardiac fibroblast revealed that under normal conditions the HspB1 was distributed throughout the cytoplasm of the cells. However, after heat shock stimuli, a large proportion of HspB1 was translocated to the nucleus, developing nuclear granules. In the same study the investigators transiently expressed the human wild type HspB1 in Rat-1 cells. After exposing the cells to heat shock, the investigators found a fraction of the human HspB1 concentrated in stress granules in the cell nucleus, thus revealing that the human HspB1 had the same behavior as the rat homologous (68).

The western blot showing the results for GFP and HspB1 expression were also a key to validate this reporter system. For GFP expression in transient cells there were no expression of GFP (32kDa) in almost all conditions tested, except for the cells with pcDNA3.1 GFP plasmid that showed expression of GFP; and the appearance of band in HspB1-GFP conditions (59kDa) proves the expression of the reporter fusion (Figure 13.A). Related to the HspB1 expression it was detected in every conditions tested (27kDa) and seemed to be stable with the increasing of the sodium arsenite concentration, and the 59kDa band also proved the expression the HspB1-GFP fusion reporter (Figure 13.B). However, an internal control was not used to confirm, quantify and compare these results. Some studies refer that HspB1 has a constitutive and ubiquitous expression in most of the cell types, playing roles as molecular chaperones in normal cell function. However, some stressing agents can increase HspB1 expression, such as oxidative stress, heavy metals, hormones, hypoxia and ischemia (51, 70). Some reports also refer that the environmental stress, such as treatment with arsenite (71), induces the phosphorylation of the HspB1 at three conserved serine residues, namely serine 15, 78 and 82, coinciding with the nuclear translocation. This phosphorylation occurs through the MAPKAP kinases 2 and 3, which are activated by phosphorylation by MAP p38 protein kinase (52, 62, 68). Moreover the state of phosphorylation depends on the type of the stimuli to which the cell is exposed, having different roles and biochemical functions and interacting with different substances (72).

4.1.3. Monoclonal cell lines

Stable HeLa cells were transfected with the pcDNA3.1 HspB1-GFP plasmid and monoclonal cells were selected from four clones. To evaluate the integration of the plasmid and the expression of the reporter a PCR, fluorescence microscopy and Western blot techniques were performed. PCR results were consistent with the previous PCR data from validation of the reporter system, dictating a good integration of the plasmid in HeLa stable cell lines for all of the four clones studied.

Concerning to the fluorescence microscopy results, there was a difference between the cells without sodium arsenite and those stimulated with this stress agent at concentrations of 250µM and 500µM. In the HeLa HspB1-GFP is shown cell shrinkage as

well as the appearance of the stress granules with sodium arsenite at 500µM when compared to the control, where the cells are in normal conditions. With sodium arsenite at 250µM the stress granules are visible as well as the cell dead and shrinkage, but not with the intensity as with the sodium arsenite at the 500µM. A study from Mao and Shelden in zebrafish reveals that HspB1 is constitutively expressed and its expression increases in response to environmental stimuli and proteotoxic stress (73). Another study in zebrafish used a cadmium-induced expression of the Hsp70-GFP reporter gene causing GFP fluorescence in several organs and tissues of the zebrafish, as the cadmium concentration increases (74).

The Western blot was consistent to the previous data obtained for reporter system validation. There was no expression of GFP; and the appearance of the 59kDa band proves the expression of the HspB1-GFP fusion reporter in every conditions except in the HeLa parental cells (Figure 18A). The constitutive HspB1 expression was also observed as in previous Western blot analysis for transient cells. The HspB1-GFP fusion was also shown, except for the HeLa parental cells that do not contain the plasmid (Figure 18.B). However, was not used an internal control, as tubulin, to confirm and compare the results of the control cells and those stimulated with different concentrations of the sodium arsenite.

4.2. Conclusion and Future Perspectives

In this study, an aggregation reporter system expressing the fusion of the HspB1-GFP was successfully developed and transfected in stable HeLa cells. The results proved that this fusion reporter was correctly transfected in HeLa cells and can be a good model to monitor protein aggregation in stress-induced cells, as it can see in fluorescent microscopy by the appearance of granules.

This reporter will be used to monitor protein aggregation in these cells, and to further identify the human tRNA modifying enzymes involved in protein homeostasis through siRNA screening.

As HeLa cells express HspB1 constitutively, it should be used another antibody to monitor other forms of HspB1, such as the phosphorylated form, because there are some evidences that this small heat shock protein is phosphorylated in response to extracellular stimuli (75, 76). So, with an antibody for phosphorylated HspB1, the differences between the normal and stress conditions should be more accentuated. Also an internal control, such as the β-tubulin, should be used to measure the expression of the HspB1 and GFP and to compare the expressions of the different concentrations of the sodium arsenite with the control.

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Appendix

Annex 1 – Reaction mix and cycle used for the amplification of GFP from pCS2 plasmid.

Reaction Mix (for 1 reaction)		
Water	37µL	
10X pfu Buffer with Magnesium Sulfate ($MgSO_4$)	5µL	
dNTP mix (10µM)	1µL	
Reverse primer (10µM)	1,25µL	
Forward primer (10µM)	1,25µL	
DNA (100ng)	1µL	
Pfu	1µL	
PCR Cycle		
Initial denaturation – 95ºC, 4 minutes		
Denaturation – 95ºC, 1 minute		35 cycles
Annealing – 61ºC, 30 seconds		
Extension – 72ºC, 4 minutes		
Final extension – 72ºC, 10 minutes		

Annex 2 – Ligation reactions.

Reagents	**Negative Control (1:0)**	**1:3**	**1:5**	**Positive Control**
T4 DNA ligase 10x buffer	2µL	2µL	2µL	2µL
T4 DNA ligase	1µL	1µL	1µL	1µL
Plasmid (pcDNA3.1)	1µL	1µL	1µL	1µL
Insert (GFP)	-	3µL	5µL	0µL
Water	16µL	13µL	11µL	16µL

Annex 3 – Reaction mix and cycle used for the amplification of HspB1-GFP and GFP from HeLa cells.

Reaction Mix (for 1 reaction)	
Water up to 50µL	40,25µL
Dream Taq Buffer	5µL
dNTP mix (10µM)	1µL
Reverse primer (10µM)	1,25µL
Forward primer (10µM)	1,25µL
DNA (100ng)	1µL
Taq	0,25µL
PCR Cycle	
Initial denaturation – 95ºC, 2 minutes	
Denaturation – 95ºC, 30 seconds	30 cycles
Annealing – 55ºC, 30 seconds	
Extension – 72ºC, 1 minute	
Final extension – 72ºC, 10 minutes	

Solutions

PBS (1L)

Reagents	Quantities
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Add H_2O to 1L and adjust pH to 7.4	

Protein Lysis Buffer for Western Blot

Reagents	Quantities
Triton X-100	50µL
HEPES	500µL
NaCl 5M	500µL
H_2O	8.95mL
ELB - 10mL	

Reagents	Quantities
ELB	4,645mL
DTT (1M)	5µL
Naf (1M)	5µL
EDTA	20µL
EGTA	20µL
Na_3VO_4	50µL
5 Aliquots of 955µL	
On the moment of use add 25L of PMSF (100mM) and 20µL of cOmplete™ EDTA-free Protease Inhibitor Cocktail tablets to each aliquot.	

Two Resolving gels for Western Blot (10%)

Reagents	Quantities
distilled H_2O	3.6mL
Tris HCl 1,5M pH8.8	3.75mL
40% Acrilamide 29:1	2.5mL
10% SDS	100µL
10% APS	100µL
TEMED	10µL

Two Stacking gels for Western Blot (4%)

Reagents	Quantities
distilled H_2O	3.646mL
Tris HCl 0.625M pH6.8	1mL
40% Acrilamide 29:1	0.5mL
10% SDS	50µL
10% APS	50µL
TEMED	10µL

10X Running Buffer for SDS-PAGE

Reagents	Quantities
Tris	30.2g
Glycine	144g
SDS	10g
Dissolve in 1L of H_2O	

TBS 10X

Reagents	Quantities
Tris	30g
NaCl	80g
KCl	2g
Add 1L of H_2O and adjust pH to 7.6	

Tris-Acetate-EDTA (TAE) Buffer (50X)

Reagents	Quantities
Tris	40mM
EDTA	2mM
Acetic Acid	20mM
Adjust pH to 8.5 and dilute with MiliQ H_2O	

Supplemental Data – Sequencing Results

The sequencing results are supplemental files that were sent together with the thesis and open with the BioEdit program.

1. Alignment of the plasmid with the HspB1 Forward primer:

Alignment pcDNA3.1 HspB1GFP HspB1_Fw.gb

2. Alignment of the plasmid with the HspB1 Forward Middle primer:

Alignment pcDNA3.1 HspB1GFP HspB1_middle.gb

3. Alignment of the plasmid with the GFP Forward primer:

Alignment GFP_Fw.gb